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# Characterization of *Bacteroides fragilis* Hemolysins and Regulation and Synergistic Interactions of HlyA and HlyB

Kirstin P. Robertson,† C. Jeffrey Smith, Andrea M. Gough, and Edson R. Rocha*

*Department of Microbiology and Immunology, East Carolina University Brody School of Medicine, Greenville, North Carolina*

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**This study describes the presence of 10 hemolysin orthologs in the genome of the opportunistic human anaerobic pathogen *Bacteroides fragilis*, which is currently classified as a nonhemolytic bacterium. The hemolysins were designated HlyA through HlyI plus HlyIII. All cloned hemolysin genes were able to confer hemolytic activity to a nonhemolytic *Escherichia coli* strain on blood agar plates. Interestingly, HlyH was found to be present in the genome of the *B. fragilis* NCTC9343 strain but absent in strains 638R, YCH46, and *Bacteroides thetaiotaomicron* VPI-5482. The hemolysins HlyA, HlyB, and HlyIII were selected for further characterization. HlyA, HlyB, and HlyIII were cytolytic to erythrocytes on liquid hemolytic assay. When *hlyA* and *hlyB* were expressed together in a nonhemolytic *E. coli* strain, the strain showed enhanced hemolytic activity on blood agar plates. Further analysis revealed that HlyA and HlyB have synergistic hemolytic activity as detected by the liquid hemolytic assay. In addition, the two-component hemolysins HlyA and HlyB form a protein-protein complex in vivo as determined by bacterial two-hybrid system assay. The *hlyB* and *hlyA* genes are organized in an operon that is coordinately regulated by iron and oxygen. Northern blot hybridization analysis revealed that *hlyBA* were expressed as a bicistronic mRNA induced approximately 2.5-fold under low-iron conditions and repressed in iron-rich medium. The normal iron-regulated expression of *hlyBA* mRNA was lost in the *furA* mutant strain. In contrast, the *hlyA* gene was also expressed as a single mRNA in iron-rich medium, but its expression was reduced approximately threefold under low-iron conditions in a Fur-independent manner. This suggests that *hlyA* alone is regulated by an unidentified iron-dependent regulator. Moreover, the expression levels of *hlyBA* and *hlyA* were reduced about threefold following oxygen exposure and treatment with hydrogen peroxide. Taken together, these results suggest that iron and oxidative stress have an effect on the control of *hlyBA* and *hlyA* transcriptional levels. A *hlyBA* mutant was constructed, and its hemolytic activity was greatly diminished compared to those of the *hlyIII* mutant and parent strains. In addition, the *hlyBA* mutant had a significant modification in colony morphology and growth deficiency compared to the parent strain. The implications of these findings for the pathophysiology of *B. fragilis* in extraintestinal infections and competition in ecological systems for this organism are discussed.**

In the human colon (where at least 500 species of bacteria have been reported to reside), *Bacteroides* species account for about 30 to 40% of the total bacteria present in some people (20, 26, 43, 54). *Bacteroides* spp. are also important opportunistic human pathogens frequently isolated from anaerobic infections (11). Within the *Bacteroide*s family, *Bacteroides fragilis* accounts for about 0.5% to 1% of total *Bacteroides* found in the human large intestinal tract (11). Despite its low frequency as a component of the intestinal microflora, *B. fragilis* is by far the number one anaerobe isolated from anaerobic infections. It accounts for 50 to 70% of all anaerobes isolated from human infections, such as intra-abdominal infections, infections of the female genital tract, deep wounds, brain abscesses, and bacteremia (4, 5, 11).

Although the whole arsenal of *B. fragilis* virulence determinants is unclear, certain factors, such as capsular polysaccharides, microbial adherence, production of proteases and neuraminidase, and inhibition of phagocytosis, are considered important (43). The most studied *B. fragilis* virulence factor associated with pathogenicity is the production of eight distinct capsular polysaccharide complexes and their relation to abscess formation (14, 15, 21). Though adherence, lipopolysaccharide, and the production of neuraminidase, enterotoxin, and proteolytic enzymes might play a role in *B. fragilis* pathogenicity, they are not currently recognized to be strong determinants of virulence (43).

There are other factors that are considered important in infections by aerobes and facultative bacteria, but they have been given little attention as far as their role in the pathogenesis of *Bacteroides* spp. is concerned. One such factor is the ability of *B. fragilis* to produce hemolysins or cytolysins. Hemolysins have been reported to be powerful virulence determinants in both gram-positive and gram-negative bacteria (24, 38, 51). Hemolysins are cytotoxic proteins that target cell membranes, and their mechanisms of damaging membrane integrity can be classified into three major groups: enzymatic activity, pore-forming cytolysin, or surfactant (51). Many microbial hemolysins offer an advantage to the bacteria by lysing and killing incoming leukocytes, thus promoting survival of the microbe by not only weakening the immune system but also by gaining access to nutrients (24). Moreover, bacterial cytolysins/hemo-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, East Carolina University Brody School of Medicine, Biotechnology Bldg., Room 130, 600 Moye Blvd., Greenville, NC 27834. Phone: (252) 744-9563. Fax: (252) 744-3535. E-mail: rochae@ecu.edu.

† Present address: Department of Microbiology, School of Biological Sciences, University of Reading, Reading, United Kingdom.

lysins play an important role in the equilibrium control of the microbial ecosystem population associated with eukaryotic hosts (9).

To date, no hemolysin has been characterized in *B. fragilis* despite the fact that rare strains have a hemolytic phenotype (18, 19). Nevertheless, a search of the *B. fragilis* genome sequence available at http://www.sanger.ac.uk/Projects/B_fragilis/ revealed the presence of 10 genes with homology to other bacterial hemolysins (K. P. Robertson, C. J. Smith, and E. R. Rocha, Abstr. 104th ASM Gen. Meet. Am. Soc. Microbiol. 2004, abstr. B-042, 2004). These findings were intriguing because this anaerobic pathogen is classified as a nonhemolytic bacterium (18, 19, 53). To clarify this apparent contradiction, putative hemolysin genes found in the *B. fragilis* genome were cloned and found to confer a hemolytic phenotype in a nonhemolytic *Escherichia coli* strain (K. P. Robertson, C. J. Smith, and E. R. Rocha, Abstr. Anaerobe 7th Biennial Cong. Anaer. Soc. Am., abstr. II-O2, 2004). More recently, independent studies have confirmed our observation that there is an extensive number of putative hemolysin homologues in the annotated genome sequences of *B. fragilis* NCTC9343 and YCH46 (7, 22). Interestingly, this extensive number of putative hemolysin genes does not correlate with the "nonhemolytic" characteristic phenotype of most clinical and laboratory strains of *B. fragilis* (18, 19, 53). Therefore, these findings prompted us to characterize these putative hemolysin genes in order to confirm their hemolytic /cytolytic properties and role in *B. fragilis* pathophysiology. It is important to mention that the only other cytotoxin that has been studied in *B. fragilis*, fragilysin, is a Zn-metalloprotease enterotoxin that is cytotoxic only to the human intestinal carcinoma cell line HT29/C1. It specifically cleaves E-cadherin of the zone adherens (40). Moreover, fragilysin is not membranolytic to eukaryotic cells (50), nor does it seem to be a virulence factor for extraintestinal infections (12). These properties are much different than the characteristics of hemolysins mentioned above.

In this study we report the characterization of *B. fragilis* hemolysins HlyA, HlyB, and HlyIII. We also show that HlyA and HlyB are two-component cytolysins that act together to enhance their respective singular hemolytic activities against erythrocytes. The *hlyB* and *hlyA* genes are organized in an operon whose expression is differentially regulated by oxygen and iron availability. In addition, we show that a *hlyBA* mutant has a diminished hemolytic activity, altered colony morphology, and a growth defect in vitro.

## MATERIALS AND METHODS

**Strains, media, and growth conditions.** *B. fragilis* strains used in this study are listed in Table 1. *B. fragilis* strain NCTC9343 is isogenic to ATCC 25285, and henceforth ATCC 25285 was used in this study for experimental purposes when required. Strains were routinely grown on BHIS (brain heart infusion supplemented with L-cysteine, hemin, and $NaHCO_3$) agar. Rifamycin (20 μg/ml), 100 μg/ml gentamicin, 5 μg/ml tetracycline, 10 μg/ml erythromycin, and 25 μg/ml cefoxitin were added to the media when required. BHIS-blood agar plate (BAP) is BHIS agar supplemented with 5% defibrinated blood from sheep, horses, rabbits, cows (Gemini Bio-Products, Woodland, CA), or adult human volunteers. For some experiments, bacteria were grown on a modified defined medium supplemented with 5% defibrinated blood (DM-BAP) and on a semidefined medium as previously described (33). *E. coli* strains were inoculated in Luria-Bertani agar (L-agar) supplemented with 40 μg/ml isopropyl-ß-D-thiogalactopyranoside (IPTG) and 100 μg/ml ampicillin. Human, horse, rabbit, sheep, or bovine defibrinated blood (5%) (Gemini Bio-Products, Woodland, CA) was added when appropriate. Glucose (0.2%) was added to L-agar for anaerobic growth of *E. coli*.

**Cloning of hemolysin genes.** The hemolysin genes listed in Table 1 were amplified by PCR using oligonucleotide primers designed to amplify the entire open reading frame (ORF) plus an additional 15 to 20 nucleotides (nt) upstream of the translation start site. Restriction sites were incorporated in the oligonucleotide primers for direct cloning of the PCR products into the cloning vector pUC19 in the same orientation as *lacZ*. *hlyA* through *hlyG* and *hlyIII* were amplified from the *B. fragilis* 638R genome, while *hlyH* was amplified from the ATCC 25285 genome. Plasmids were transformed into nonhemolytic *E. coli* strains DH10B and screened for hemolytic activity on Luria-Bertani agar plates incubated at 37°C for 24 to 48 h. In some experiments *E. coli* YMZ19 was used as the host strain to confirm that the cloned genes were not interfering with endogenous *E. coli* hemolytic activity. Hemolytic activity was detected by the appearance of clear hemolytic zones around the bacterial colonies compared to the control host strain carrying vector alone.

**Measurement of hemolytic activity.** Bacteria were harvested by centrifugation at 10,000 × *g* for 10 min. The cell pellet was washed with phosphate-buffered saline (PBS) (50 mM phosphate buffer, pH 7.4, 150 mM NaCl) and resuspended in 5 ml PBS. Cell lysates were obtained by using a French press. Whole cells and cell debris were separated at 10,000 × *g* for 30 min. Lysates were maintained at 4°C or stored at −70°C until needed. For liquid hemolytic assays, a protocol modified from the methods of Bernheimer (1) and Rowe and Welch (38) was used. Briefly, sheep red blood cells (RBCs) were washed with PBS and centrifuged at 400 × *g* for 5 min. Washes were repeated until the supernatant was visibly clear of hemoglobin. Erythrocyte suspensions were adjusted to 1% with PBS supplemented with 0.1% bovine serum albumin. Crude extracts were adjusted to approximately 2 mg/ml of protein. Then, 0.5 ml of this solution was incubated with 0.5 ml of the 1% erythrocyte suspension at 37°C. Samples were centrifuged at 120 × *g* for 7 min to remove undamaged RBCs. The concentration of released hemoglobin is estimated by reading absorbance at 545 nm in a spectrophotometer against a control background lysis solution (0.5 ml erythrocyte suspension with 0.5 ml PBS). A 100% hemolysis standard was obtained by mixing 1 volume of distilled water containing 0.04% saponin and 1 volume of 1% RBC suspension.

**Construction of *hlyBA* and *hlyIII* deletion mutants.** A 1.43-kb chromosomal DNA fragment upstream from *hlyB*, including the first 57 nt within the N-terminal region, was amplified by PCR and cloned into the unique BamHI site of the *E. coli-Bacteroides* shuttle suicide vector pFD516 (42). Subsequently, a 1.68-kb DNA fragment downstream from *hlyA*, containing the last 155 nt of the *hlyA* C-terminal region, was amplified by PCR and cloned into the unique EcoRI site of the new construct pER-61. A 2.4-kb SstI fragment containing the tetracycline resistance gene *tetQ* was cloned into the unique SstI site of pER-61 to replace the internal 1.65-kb DNA fragment deleted from the *hlyBA*. The new plasmid pER-62, containing the Δ*hlyBA*::*tetQ* construct, was mobilized from *E. coli* DH10B into *B. fragilis* 638R by triparental filter mating protocols (41). Transconjugants were selected on BHIS agar containing 20 μg of rifamycin per ml, 100 μg of gentamicin per ml, and 5 μg of tetracycline per ml. Determination of sensitivity to either tetracycline or erythromycin was carried out to identify recombinants that were tetracycline resistant and erythromycin sensitive. Southern blot analysis was used to confirm the double-crossover genetic allele exchange of pER-62 into the *B. fragilis* chromosome. A transconjugant, BER-41, containing the Δ*hlyBA*::*tetQ* construct inserted into the *B. fragilis* 638R chromosome was selected for further studies.

To construct the *hlyIII* deletion mutant, a 520-bp internal DNA fragment from *hlyIII* was deleted and replaced by a 2.1-kb cefoxitin resistance gene, *cfxA*. Briefly, a 2.1-kb BamHI/EcoRI *cfxA* DNA fragment was cloned into the unique BamHI and EcoRI sites of the suicide vector pFD516. Then, a 1.52-kb DNA fragment upstream and a 1.60-kb DNA fragment downstream of the *hlyIII* gene were cloned, respectively, into the unique SphI/BamHI and EcoRI restriction sites of the pFD516/*cfxA* construct. The new plasmid, pER-64, carrying the Δ*hlyIII*::*cfxA* construct was mobilized into *B. fragilis* as described above. Transconjugants were selected on BHIS agar containing 20 μg of rifamycin per ml, 100 μg of gentamicin per ml, and 25 μg of cefoxitin per ml. Sensitivity to either cefoxitin or erythromycin was carried out to identify recombinants that were cefoxitin resistant and erythromycin sensitive as described above. A *B. fragilis* transconjugant, BER-45, containing the Δ*hlyIII*::*cfxA* construct inserted into the chromosome by double-crossover allelic recombination was selected for further analysis. The *hlyBA*::*tetQ hlyIII*::*cfxA* double mutant strain, BER-46, was obtained by mobilizing pER45 into strain BER-41 as described above.

**Construction of a *furA* null mutant.** Briefly, a 275-bp EcoRV/NruI DNA fragment containing the first 25 N-terminal codons and 200 bp upstream of the

TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Relevant phenotype or genotype or description[a] | Source or reference[b] |
|---|---|---|
| *B. fragilis* | | |
| ATCC 23745 | Clinical isolate, pleural fluid | ATCC |
| ATCC 25285 | Clinical isolate, appendix abscess (same as NCTC 9343) | ATCC |
| VPI 2393 | Unknown | VPI |
| 638R | Clinical isolate, Rif$^r$ | 30 |
| BER-2 | 638R Δ*furA* Rif$^r$ Cfx$^r$ | This study |
| BER-41 | 638R Δ*hlyBA*::*tetQ* Rif$^r$ Tet$^r$ | This study |
| BER-45 | 638R Δ*hlyIII*::*cfxA* Rif$^r$ Cfx$^r$ | This study |
| BER-46 | 638R Δ*hlyBA*::*tetQ* Δ*hlyIII*::*cfxA* Rif$^r$ Tet$^r$ Cfx$^r$ | This study |
| *E. coli* | | |
| DH10B | Cloning host strain | Invitrogen |
| YMZ19 | MC1061 *clyA*::*kan* | 47 |
| Plasmids | | |
| pUC19 | Cloning vector, Amp$^r$ | Invitrogen |
| pGEM-T | Cloning vector | Promega |
| pFD516 | Suicide vector, derived from deletion of pBI143 in pFD288 (Tet$^r$) (Sp$^r$) (Erm$^r$) | 41 |
| pER-2 | 1,047-bp dsDNA fragment amplified from 638R chromosome containing *hlyF* ORF was cloned into the SphI/BamHI sites of pUC19 | This study |
| pER-3 | 1,445-bp dsDNA fragment amplified from 638R chromosome containing *hlyE* ORF was cloned into the SphI/BamHI sites of pUC19 | This study |
| pER-9 | 3.2-kb DNA fragment from 638R containing a 363-bp in-frame deletion in *furA* plus 2.1-kb *cfxA* gene cloned into the intergenic region cloned into pGEM-T | This study |
| pER-10 | 5.5-kb DNA fragment from pER-9 containing the Δ*furA* construct was cloned into the BamHI/SstI sites of pFD516 | This study |
| pER-11 | 1,170-bp dsDNA fragment amplified from 638R chromosome containing *hlyA* ORF was cloned into the BamHI/SstI sites of pUC19 | This study |
| pER-12 | 933-bp dsDNA fragment amplified from 638R chromosome containing *hlyB* ORF was cloned into the BamHI/SstI blunted sites of pUC19 | This study |
| pER-13 | 1,477-bp dsDNA fragment amplified from 638R chromosome containing *hlyG* ORF was cloned into the BamHI/SstI sites of pUC19 | This study |
| pER-14 | 1,139-bp dsDNA fragment amplified from ATCC 25285 chromosome containing *hlyH* ORF was cloned into the BamHI/SstI sites of pUC19 | This study |
| pER-15 | 1,272-bp dsDNA fragment amplified from 638R chromosome containing *hlyC* ORF was cloned into the BamHI/SstI sites of pUC19 | This study |
| pER-16 | 363-bp dsDNA fragment amplified from 638R chromosome containing *hlyD* ORF was cloned into the BamHI/SstI sites of pUC19 | This study |
| pER-17 | 820-bp dsDNA fragment amplified from 638R chromosome containing *hlyIII* ORF was cloned into the BamHI/SstI sites of pUC19 | This study |
| pER-18 | 2,035-bp dsDNA fragment amplified from 638R chromosome containing *hlyBA* ORFs was cloned into the BamHI/SstI sites of pUC19 | This study |
| pER-61 | Suicide vector containing a Δ*hlyBA* construct in pFD516 | This study |
| pER-62 | 2.4-kb SstI fragment containing the *tetQ* gene cloned into the unique SstI site of pER-61 to make a Δ*hlyBA*::*tetQ* construct in pFD516 | This study |
| pER-64 | Suicide vector containing a Δ*hlyIII*::*cfxA* construct in pFD516 | This study |

[a] Rif$^r$, rifampin resistance; Cfx$^r$, cefoxitine resistance; Tet$^r$, tetracycline resistance; Amp$^r$, ampicillin resistance; Sp$^r$, spectinomycin resistance; Erm$^r$, erythromycin resistance. Antibiotic resistance expression in *E. coli* is indicated by drug resistance phenotypes shown in parentheses. dsDNA, double-stranded DNA.

[b] ATCC, American Type Culture Collection (Manassas, VA); VPI, Anaerobe Laboratory, Virginia Polytechnic Institute and State University (Blacksburg, VA).

*furA* promoter region was ligated in frame to a 1.7-kb NruI/SstI DNA fragment containing the last 17 *furA* codons and downstream region into pGEM-T (Promega, Madison, WI). The null mutation construct contained an in-frame deletion of 363 nt from the *furA* gene. Next, a 2.1-kb BamHI/blunted EcoRI *cfxA* gene was cloned into the BamHI/EcoRV sites of the new construct. Then, a 1.5-kb BamHI/BglII DNA fragment upstream of the *furA* promoter region was cloned into the BamHI site of the new construct pER-9. A 5.5-kb BamHI/SstI DNA fragment from pER-9 was cloned into the unique BamHI/SstI sites of the suicide vector pFD516. The new plasmid, pER10, was mobilized into *B. fragilis* 638R as described above. The mutant strain BER-2 containing the Δ*furA* construct inserted into the chromosome by double-crossover allelic recombination was grown under iron-replete and iron-limiting conditions. Total RNA was extracted as described below. Real-time reverse transcription-PCR of *feoAB* transcripts and *feoAB*::*xylB* transcriptional fusion analysis were carried out to determine whether strain BER-2 lost the normal iron regulation uptake mechanism controlled by the ferric uptake regulator FurA in other bacteria (23). Both methods showed that *feoAB* mRNA expression was no longer repressed under high-iron conditions compared to the parent strain, confirming the mutation and loss of normal iron-responsive FurA regulation (data not shown).

**Bacterial two-hybrid system assay.** *hlyB* was cloned into the "bait" vector pBT (BacterioMatch II; Stratagene, La Jolla, CA) in frame with λ*c*I. *hlyA* was cloned into the "prey" vector pTRG in frame with the α-subunit of RNA polymerase according to the manufacturer's instructions. The new constructs were cotransformed into the *E. coli* two-hybrid system reporter strain derived from XL1-Blue MRF′ kan [Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*$^q$*ZΔM15* Tn*5* (Kan$^r$)] containing the *HIS3-aadA* reporter cassette (Stratagene) and plated on selective screening medium. The selective screening plate is histidine-dropout M9 agar supplemented with 0.5 mM IPTG, 10 μg/ml tetracycline, 25 μg/ml chloramphenicol, and 5 mM 3-amino-1,2,4-triazole. Culture media were prepared according to the manufacturer's instructions. Transformants from selective medium were grown on dual-selective medium to confirm the interaction of the loaded "prey" and loaded "bait" constructs by the activation of the dual reporter system assay. Dual-selective

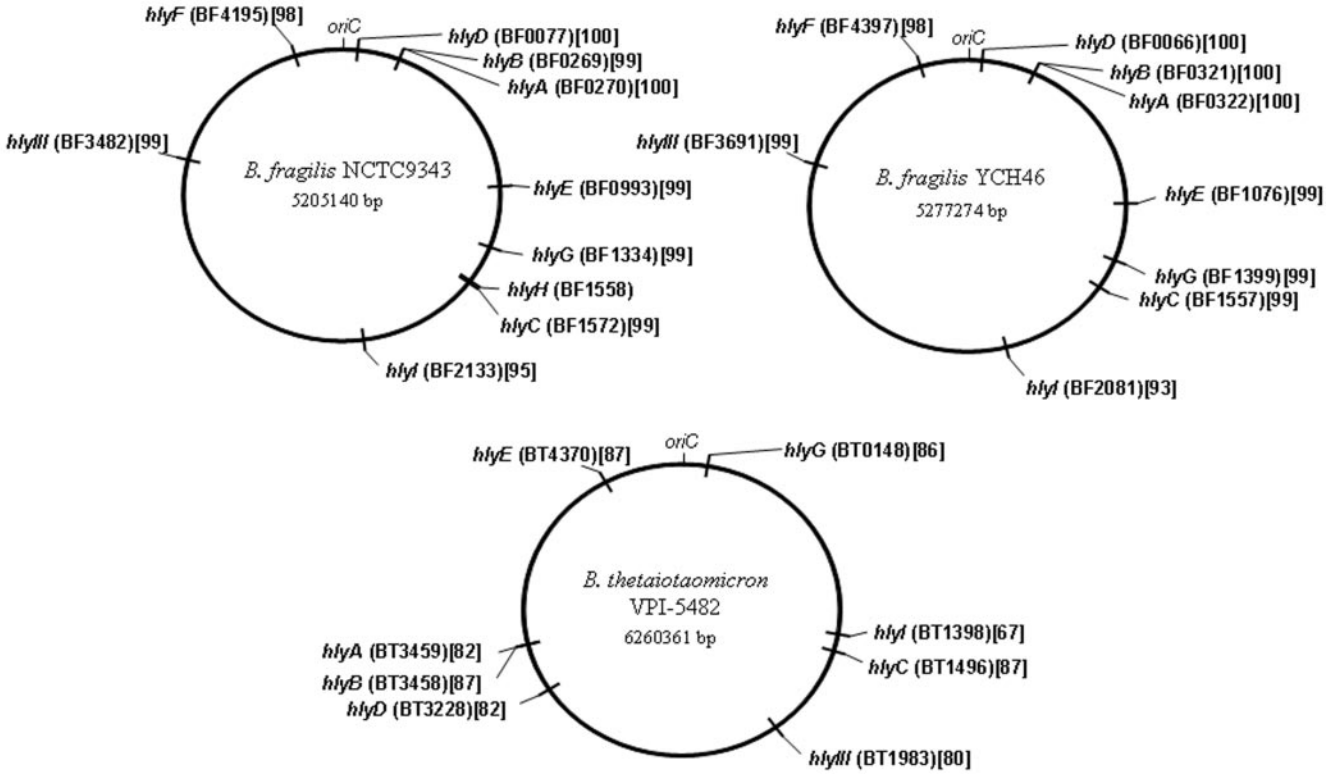


FIG. 1. Circular map showing the chromosomal locations of putative hemolysins within *B. fragilis* and *B. thetaiotaomicron* strains. The peptide sequence for each hemolysin from strain 638R was used to identify the homologous peptide and respective locus tag number in *B. fragilis* NCTC9343 and YCH46 and *B. thetaiotaomicron* VPI-5482. The locus tags for strain 638R are not shown, because the genome annotation has not yet been released to public domain databases though the complete genome sequence is available at http://www.sanger.ac.uk/Projects/B_fragilis/. The locus tag locations shown in parentheses were retrieved from references 7, 22, and 55 and from websites and an FTP site (http://www.sanger.ac.uk/Projects/B_fragilis/, ftp://ftp.sanger.ac.uk/pub/pathogens/bf/, and http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?db=nucleotide&val=60491031). The percentage of amino acid identity of each gene product compared to that in strain 638R is shown in brackets. The genome length (in nucleotides) is depicted in each panel below the respective strain name.

screening plates are the same as selective medium but 12.5 μg/ml streptomycin is added. Self-activation controls are the *E. coli* two-hybrid system reporter strain carrying the following constructs: control 1, empty "bait" (pBT alone) cotransformed with loaded "prey" (pTRG/HlyA); control 2, loaded "bait" (pBT/HlyB) cotransformed with empty "prey" (pTRG alone).

**RNA extraction and Northern blot hybridization.** Bacteria were grown in semidefined medium (SDM) as previously described (33). Hemin was replaced by 5 μg/ml protoporphyrin IX when required. Addition of 50 μM 2,2′-bipyridyl and 100 μM desferrioxamine was used to restrict iron availability in SDM. Ferrous sulfate at 100 μM was added as indicated in the text. For oxidative stress experiments, cultures were grown to an $A_{550}$ of 0.3 and treated with 50 μM $H_2O_2$ for 5 min prior to total RNA extraction. To induce oxygen stress, cultures were split in half, one half was kept anaerobically and the other half was shaken aerobically at 250 rpm at a volume/flask ratio of 1/5 as previously described. Total RNA extraction and Northern blot analysis of mRNA were carried out as previously described (32), and internal fragments of *hlyA* and *hlyB* were used as specific probes. Densitometry analysis of the autoradiograph was normalized to the relative intensity of total 23S and 16S rRNA detected on the ethidium bromide-stained agarose gel to correct for any loading differences.

## RESULTS

Characterization of *B. fragilis* hemolysins was initiated following the release of the *B. fragilis* NCTC9343 (ATCC 25285) and 638R genome sequences at http://www.sanger.ac.uk/Projects/B_fragilis/. A search in the NCTC9343 genome revealed the presence of 10 putative genes with homology to known bacterial hemolysin genes available in the GenBank database. Henceforth they are designated *hlyA* through *hlyI* plus *hlyIII*. Strain 638R has all the same hemolysin genes as NCTC9343 except for *hlyH* (Fig. 1 and data not shown). Moreover, *hlyH* was found to be part of a 25-kbp region found in the NCTC9343 strain but absent in strains 638R and YCH46. The *hlyH* chromosomal region is flanked by insertion sequence elements, and we speculate that it might be an unidentified putative pathogenicity island unique to NCTC9343 strain (unpublished data). The release of the complete genomic annotations of strains NCTC9343 (7) and YCH46 (22) confirmed our previous assumption that putative hemolysins are indeed present in the *B. fragilis* genome.

A comparison of the genetic organization of the putative hemolysins in *B. fragilis* species revealed that *hlyIII* and *hlyA* through *hlyI* are located in similar positions in the chromosomes of strains NCTC9343, 638R, and YCH46 (Fig. 1 and data not shown). In contrast, the loci of hemolysin orthologs in *Bacteroides thetaiotaomicron* VPI-5482 (55) are found at different locations compared to *B. fragilis* relative to the chromosome origin of replication *oriC* (Fig. 1). This suggests that there have been major genetic rearrangements between these two species. Both *hlyF* and *hlyH* homologues are missing in the *B. thetaiotaomicron* VPI-5482 genome.

**Hemolytic activity on BAP.** When strains ATCC 25285, 638R, ATCC 23745, and VPI 2393 were grown on DM-BAP,

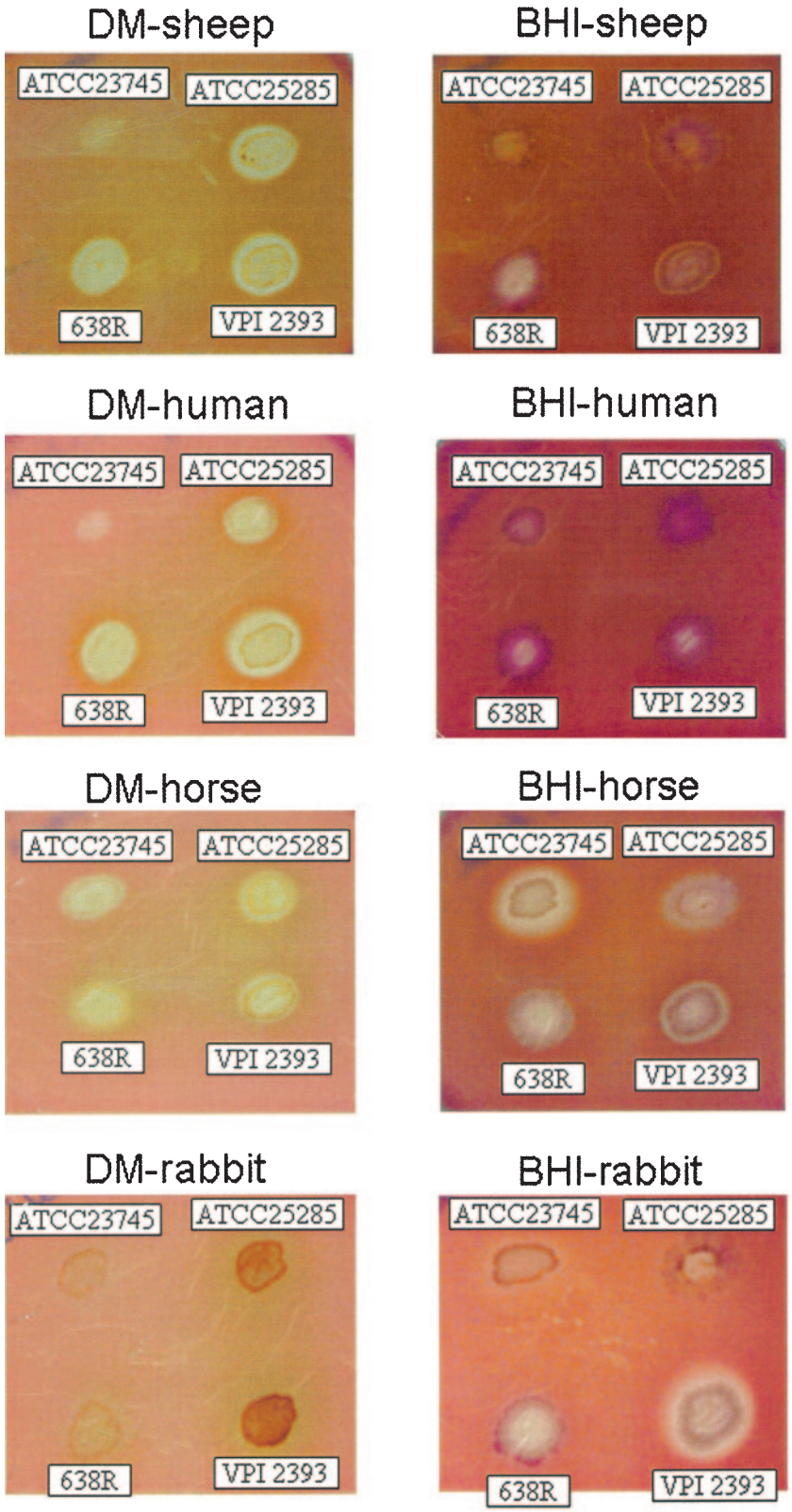


FIG. 2. Hemolytic activity of *B. fragilis* strains grown on defined medium (DM) and BHIS agar supplemented with 5% defibrinated blood. Plates were incubated anaerobically at 37°C for 5 days. Strain designations and animal blood source are shown in each panel.

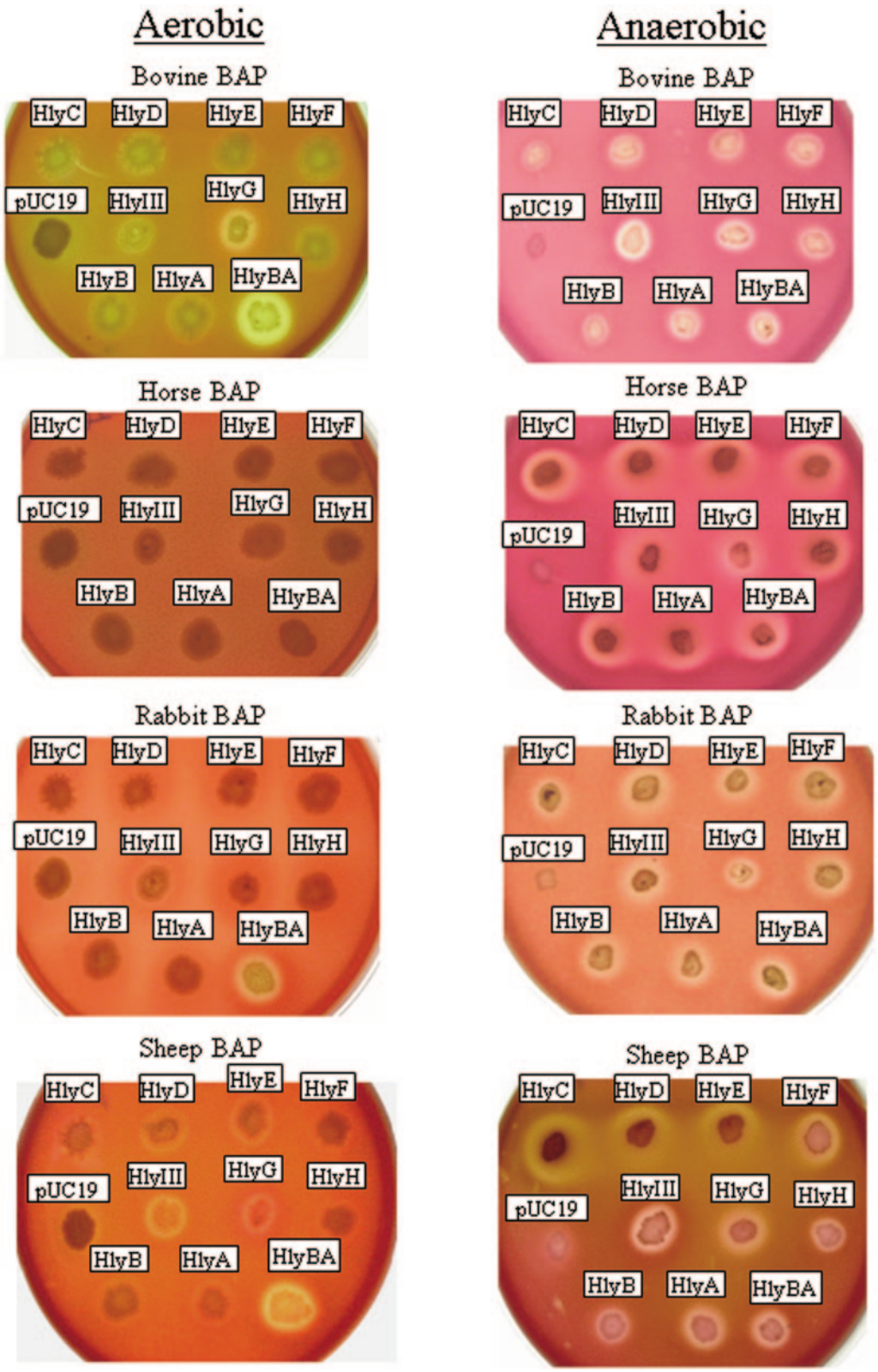


FIG. 3. Hemolytic activity of *E. coli* DH10B transformed with pUC19 carrying *B. fragilis* hemolysin genes. Bacteria were grown aerobically on L-agar medium supplemented with 5% defibrinated blood (BAP) and 0.5 mM IPTG. For anaerobic growth, media were supplemented with 0.2% glucose. *E. coli* carrying pUC19 alone was used as a control vector. All genes were amplified from *B. fragilis* 638R except for *hlyH* which was amplified from ATCC 25285 strain. Hemolytic activity was determined by the appearance of a clear zone around the growth (beta-hemolysis) or the appearance of a greenish hemolytic zone around the colonies (alpha-hemolysis).

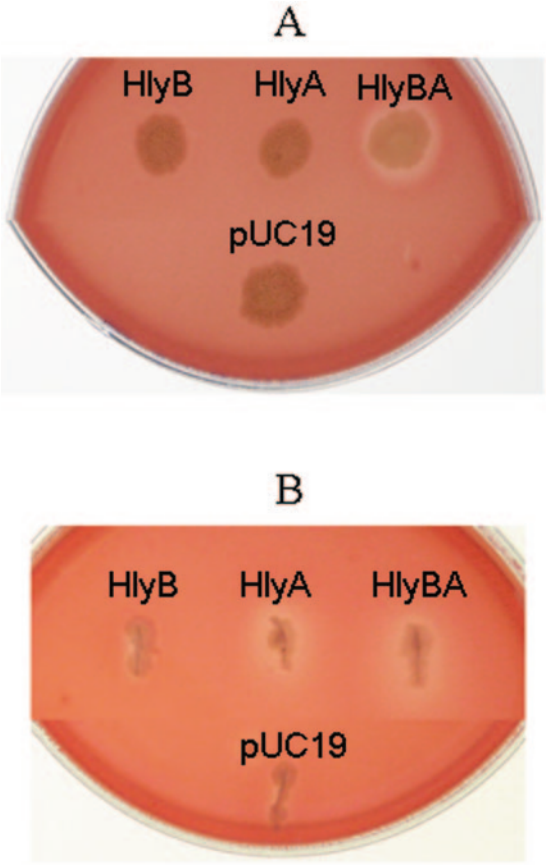


FIG. 4. Hemolytic activity of *E. coli* YMZ19 (*clyA*::*kan*) expressing *B. fragilis* hemolysins HlyB, HlyA, and HlyBA and the vector pUC19 alone. Cultures were inoculated on L-agar plates containing 5% defibrinated human blood plus 0.5 mM IPTG, 100 μg/ml ampicillin, and 50 μg/ml kanamycin. (A) Bacteria inoculated on top of the agar. (B) Bacteria were stabbed through the blood agar.

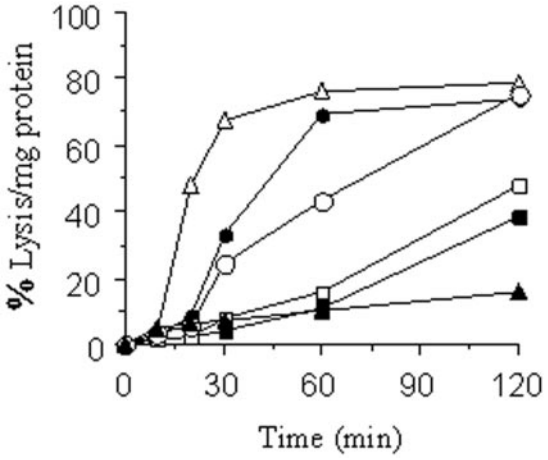


FIG. 5. Liquid hemolytic assay. Crude extracts of *E. coli* carrying HlyIII, HlyBA, and HlyB were used to determine hemolytic activity. *E. coli* carrying vector pUC19 alone was used as a control. Sheep erythrocytes were used in the liquid hemolytic assay as target cells. The release of hemoglobin was measured in the supernatant at $A_{540}$. Symbols: △, HlyIII; ●, HlyBA; □, HlyA; ■, HlyB; ○, HlyA plus HlyB; ▲, pUC19. A lysis assay was carried out by mixing 0.5 ml of crude extract, normalized to 2 mg/ml of protein, with 0.5 ml of the erythrocytes suspension (see Materials and Methods for details). Data are the averages for three experiments. All the standard errors were less than 5% and were therefore not included on the graph for clarity.

they produced beta-hemolysis after 3 to 5 days, but much weaker activity was observed on BHIS-BAP during the same period of incubation (Fig. 2). The early appearance of beta-hemolytic zones on DM-BAP compared to BHI-BAP was an indication that the production of hemolysin(s) might be under the control of a nutritional/starvation mechanism. These results suggest that the apparent lack of the hemolytic phenotype in clinical isolates of *B. fragilis* may be the consequence of a short period of incubation typically used in clinical laboratory diagnosis procedures. Strains ATCC 25285, 638R, and VPI 2393, were hemolytic for sheep, human, and horse red blood cells on DM-BAP. In contrast, strain ATCC 23745 showed strong hemolytic activity to horse RBCs but not to sheep and human RBCs (Fig. 2). Interestingly, no apparent hemolytic activity was detected on DM-BAP containing rabbit RBCs, although strains ATCC 27285 and VPI 2393 produced dark brown pigmentation. Furthermore, strains ATCC 23745 and VPI 2393 showed strong hemolytic activity against horse and rabbit RBCs on BHI-BAP. These findings suggest that the hemolytic activity produced by *B. fragilis* might be influenced by differences in strain background, the origin of the RBCs, and growth conditions.

**Cloning of hemolysin genes.** To further characterize *B. fragilis* hemolysins at a molecular level, *hlyA* through *hlyH* plus *hlyIII* were cloned, overexpressed in the host strain *E. coli* DH10B, and investigated for their ability to lyse RBCs. *hlyI* was not characterized in this study. Figure 3 shows that compared to controls with vector alone, all cloned genes conferred beta-hemolytic phenotype to *E. coli* grown under anaerobic conditions on BAP containing different animal RBCs. In contrast, there were differences in the hemolytic patterns when cells were grown under aerobic conditions. For example, horse RBCs did not show detectable hemolytic activity aerobically but were highly susceptible to hemolysis anaerobically. On bovine BAP, HlyG was beta-hemolytic under both aerobic and anaerobic conditions, while HlyIII showed alpha-hemolysis aerobically and stronger beta-hemolysis anaerobically. Interestingly, HlyB and HlyA showed alpha-hemolysis on bovine, rabbit, and sheep RBCs when expressed alone, but when expressed together, they showed ß-hemolysis, suggesting that they may act synergistically under aerobic conditions. In contrast, in the absence of oxygen, HlyB, HlyA, and HlyBA were beta-hemolytic. Taken together, these data clearly demonstrate that major differences exist in the activity of these hemolysins depending on the animal source of the RBCs and the presence or absence of oxygen.

**Analysis of HlyA and HlyB hemolytic activity in the *E. coli clyA* mutant.** At this point of the investigation, we were particularly interested in further investigating HlyA and HlyB because our findings showed that they might act synergistically (Fig. 3). Plasmids pER-11, pER-12, and pER-18 were transformed into *E. coli* strain YMZ19 deleted for its cryptic, chromosome-encoded, silent hemolysin ClyA (49). When HlyB and HlyA were expressed together in pER-18, they showed syner-

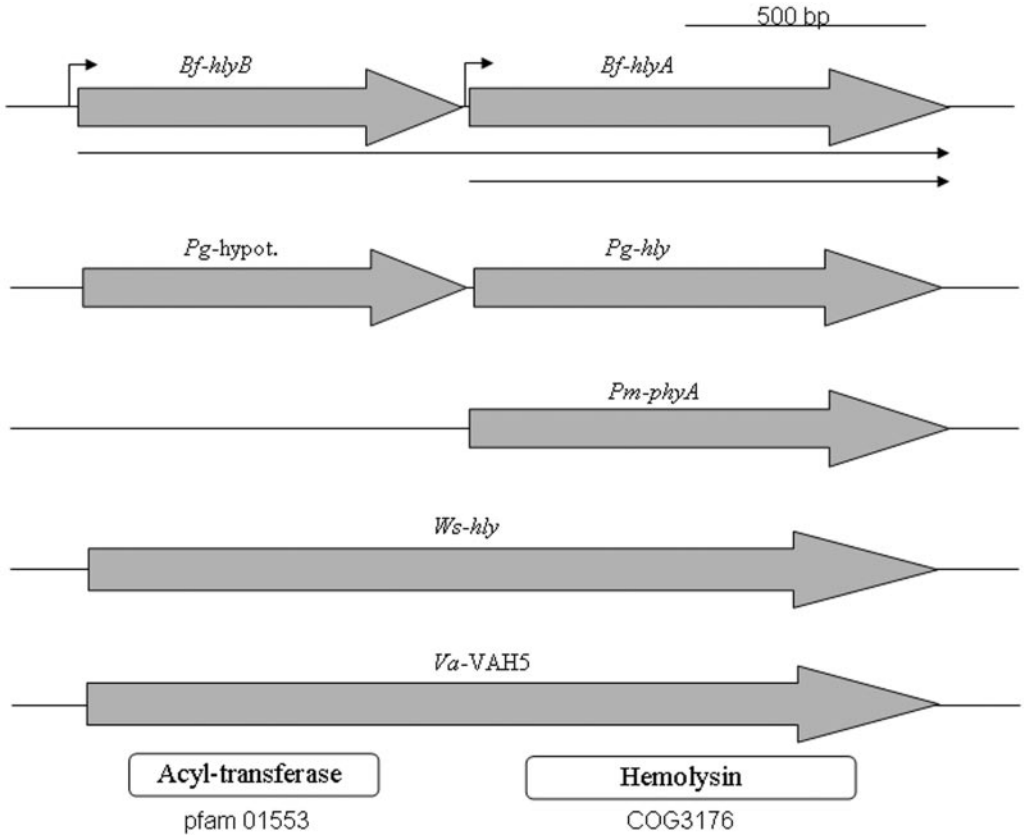


FIG. 6. Diagram showing the genetic organization of *B. fragilis* (*Bf*) hemolysin genes *hlyB* and *hlyA* with other bacterial hemolysin orthologs. *Pg*-hypot., *Porphyromonas gingivalis* W83 conserved hypothetical protein (GenBank accession no. AAQ66862); *Pg-hly*, *P. gingivalis* W83 hemolysin A (AAQ66861); *Pm-phyA*, *Prevotella melaninogenica* hemolysin PhyA (AAB88217); *Ws-hly*, *Wolinella succinogenes* DSM 1740 hemolysin (NP_908080); *Va*-VAH5, *Vibrio anguillarum* hemolysin VAH5 (AB189398). The gray arrows indicate the open reading frames and their respective direction of transcription. The thin arrows depict mRNA transcript length. The bent arrow depicts the putative promoter region derived from Fig. 9. The conserved motif domains pfam01553 (acyltransferase) and COG3176 (hemolysin) present in respective gene products are depicted as boxes underneath the panel and were obtained from a search in the collection of multiple-sequence alignments at http://www.ncbi.nlm.nih.gov/.

gistic hemolytic activity on the surface of human BAP incubated aerobically, but no activity when expressed alone compared to the vector control strain (Fig. 4). In contrast, in stabbed agar cultures, HlyBA and HlyA alone were hemolytic, while HlyB itself showed no detectable hemolysis under the same conditions compared to control vector. These findings confirm our hypotheses that HlyA and HlyB have synergistic interactions and that their activity was affected by exposure to air.

**Liquid hemolytic activity assay.** Cytotoxicity (measured by lysis of erythrocytes) of HlyBA, HlyB, HlyA, and HlyIII against sheep RBCs was determined by the liquid hemolytic assay (Fig. 5). Crude extracts of *E. coli* expressing HlyBA showed strong hemolytic activity against sheep RBCs, as 50% lysis occurred in just over 30 min of incubation. However, there was a weaker hemolytic activity in crude extracts containing HlyB or HlyA expressed alone. When crude extracts containing HlyB and HlyA were mixed together before addition to RBCs, hemolytic activity was restored similar to extracts containing HlyBA. These results correlate with previous data and add further support for the fact that HlyB and HlyA may have synergistic properties. Under the same conditions, HlyIII lysed 50% of RBCs in about 20 min at 37°C similar to lysis obtained with the HlyBA extract.

**Protein-protein interaction between HlyA and HlyB.** The alignment of HlyA and HlyB amino acid sequences with other bacterial hemolysins revealed that HlyB was homologous (34% identity and 48.5% similarity) to the N-terminal half of *Wolinella succinogenes* hemolysin and *Vibrio anguillarum* VAH5 (34), while HlyA was homologous (33% identity and 45% similarity) to the C-terminal region (Fig. 6). HlyA also showed strong homology to *Porphyromonas gingivalis* hemolysin (58% identity and 69% similarity) and *Prevotella melaninogenica* hemolysin PhyA (68% identity and 77.5% similarity). In Fig. 6 we show the genetic organization of HlyA and HlyB, which are both similar to *V. anguillarum* VAH5 and *W. succinogenes* hemolysins, indicating that they might interact as if they were a large single protein unit. These findings led us to think that HlyA and HlyB would likely have the potential to form protein-protein interactions. Thus, in order to further investigate this hypothesis, experiments using the bacterial two-hybrid system assay were carried out to determine whether HlyA and HlyB would be able to form a complex in vivo. The data shown in Fig. 7 confirm that the HlyB and HlyA form a complex in vivo.

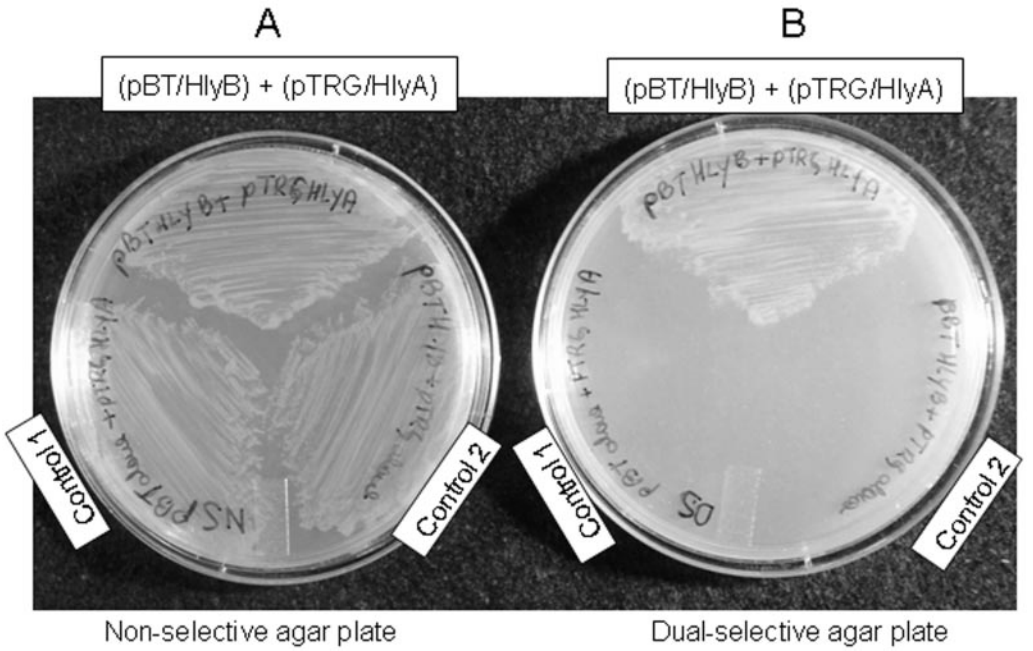


FIG. 7. *E. coli* two-hybrid system assay showing protein-protein interaction between HlyB and HlyA. *E. coli* reporter strain (Stratagene, La Jolla, CA) cotransformed with pBT-HlyB (bait) and pTRG-HlyA (prey) constructs were grown on (A) nonselective plate and (B) dual-selective plate. Self-activation controls are *E. coli* two-hybrid system reporter strain carrying the following constructs: control 1, empty "bait" (pBT alone) cotransformed with loaded "prey" (pTRG/HlyA); control 2, loaded "bait" (pBT/HlyB) cotransformed with empty "prey" (pTRG alone). See Materials and Methods for details on the bacterial two-hybrid system assay.

Strains containing both the "bait" (pBT/HlyB) and "prey" (pTRG/HlyA) constructs were able to grow on the selective and dual-selective agar plate. In contrast, when the reporter strain was carrying either empty "bait" or empty "prey," as control for self-activation, there was no growth on the selective medium. This indicates that an interaction between HlyA and HlyB occurred and activated the two-hybrid system, allowing for growth on the selective reporter medium. Taken together, the data presented above and in Fig. 3, 4, and 5 provide evidence that the enhanced hemolytic activity of HlyA and HlyB together is due to the formation of a protein-protein complex.

**Characterization of *hlyBA* and *hlyIII* deletion mutants.** When the *hlyBA* mutant strain was grown on BAP, hemolytic activity was greatly diminished compared to the parent strain (Fig. 8A). In contrast, the *hlyIII* mutant strain was not significantly different from the parent strain hemolytic zone. The *hlyBA hlyIII* double mutant had a diminished, but not abolished, hemolytic zone similar to the *hlyBA* mutant. These findings show that the HlyBA hemolysin was responsible in large part for the *B. fragilis* hemolytic activity on BAP. This also suggests that a hemolysin(s) other than HlyBA and HlyIII is still expressed and is yet to be further characterized. In addition, the *hlyBA* strain had a growth deficiency in SDM medium compared to both the parent and *hlyIII* strain (Fig. 8C). The growth rate of the *hlyBA hlyIII* double mutant was nearly identical to that of the *hlyBA* strain. No significant difference in growth rate was observed when bacteria were grown on rich broth medium (data not shown). These findings correlate with the data presented previously showing that *B. fragilis* had a strong hemolytic activity on DM-BAP compared to BHIS-BAP. Additional evidence that a disruption in HlyBA activity affects the normal physiological homeostasis of *B. fragilis* was the effect on colony morphology. When spotted on the surface of a BHIS agar plate, the *hlyBA* mutant had a significant modification in colony morphology compared to the parent strain (Fig. 8B). *hlyBA* mutant colonies were irregular and small in contrast to the circular and regular edges observed in the parent strain. Lack of HlyIII did not apparently alter *B. fragilis* colony morphology.

**Effect of iron availability on *hlyA* and *hlyB* expression.** As a first step to understanding regulation of *B. fragilis* hemolysin expression, *hlyBA* was used as a model. The *hlyBA* operon is located immediately downstream from an iron-regulated heme uptake outer membrane protein homolog similar to *V. cholerae* HutA (16; also data not shown). Therefore, it was plausible that *hlyBA* regulation was affected by iron availability. To study this, a *furA* null mutant was constructed and the expression of *hlyA* and *hlyB* mRNA was analyzed under iron-replete and iron-limiting conditions. Northern blot hybridization analysis revealed that *hlyBA* was expressed as a bicistronic mRNA and up-regulated about 2.5-fold under low-iron conditions in the parent strain. In contrast, the normal iron-regulated expression of *hlyBA* mRNA in the parent strain was no longer repressed under high-iron conditions in the *fur* mutant (Fig. 9A and B). Interestingly, *hlyA* was also expressed as a single mRNA in iron-rich medium, but *hlyA* mRNA expression was repressed approximately threefold under iron-limiting conditions in a Fur-independent manner. This suggests that *hlyBA* and *hlyA* mRNAs are differentially regulated by iron and that *hlyA* alone is also regulated by an unidentified iron-dependent regulator.

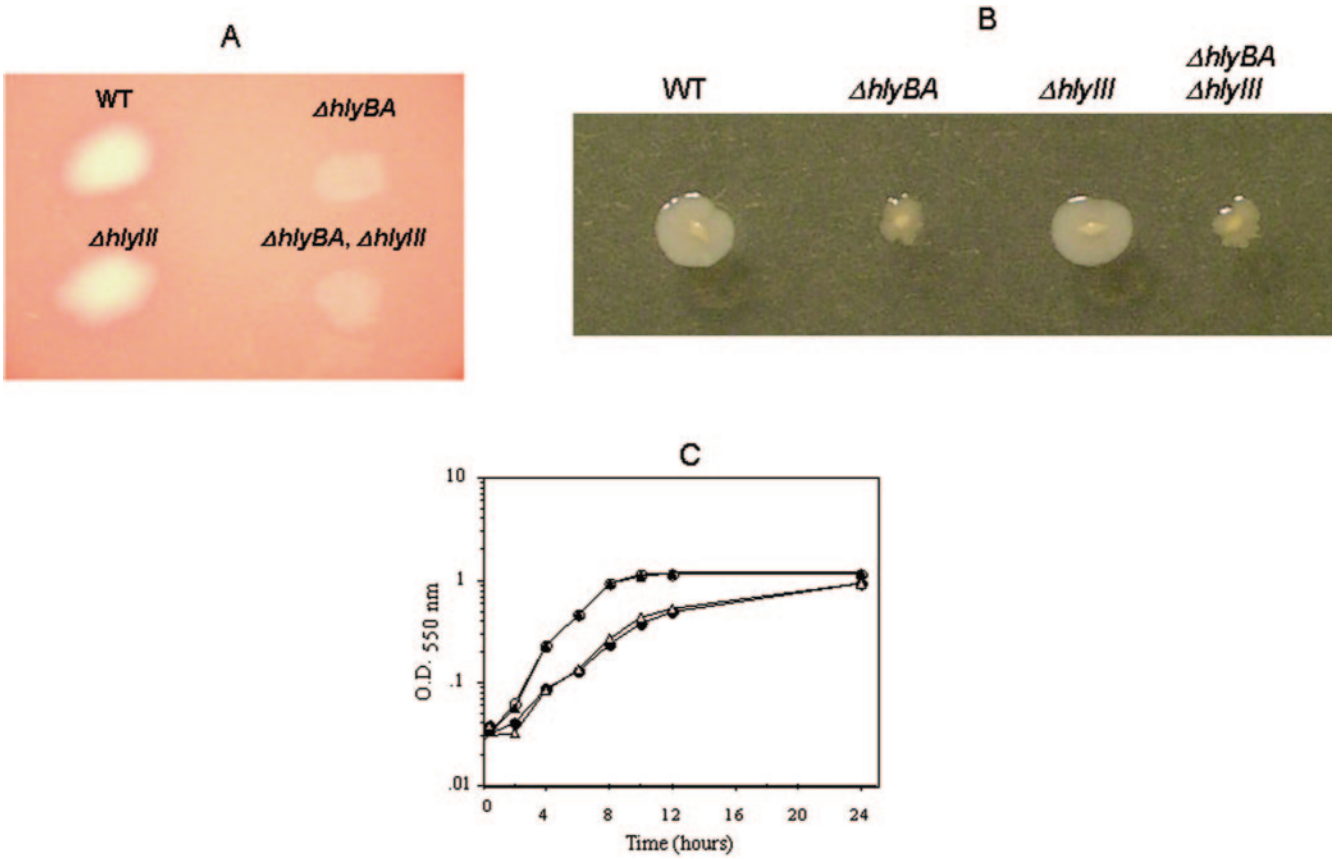


FIG. 8. Characterization of *hlyBA* and *hlyIII* mutants. A) Hemolytic activity produced by *B. fragilis* strains. Bacteria were grown on BHIS agar supplemented with 5% human defibrinated blood. Plates were incubated for 8 days anaerobically. Bacteria were removed from the agar surface with sterile cotton swab for clarity. B) Colony morphology of *B. fragilis* strains grown on agar. Each strain was inoculated on top of BHIS agar and grown under anaerobic conditions. Strain designations are depicted above the colonies in panels A and B (WT, wild-type 638R parent strain). C) Growth of *B. fragilis* strains on SDM. Bacteria were inoculated into fresh SDM from an overnight culture, and growth rate was measured at an optical density (O.D.) of 550 nm. Data presented are averages from two independent experiments. Symbols: ○, 638R parent strain; ●, Δ*hlyBA* mutant strain; ▲, Δ*hlyIII* mutant strain; △, Δ*hlyBA* Δ*hlyIII* double mutant strain.

**Effects of oxidative stress on *hlyA* and *hlyB* expression.** To determine whether HlyA and HlyB are regulated by a change in the cellular redox balance, cultures were exposed to atmospheric oxygen and to the oxidant hydrogen peroxide. Figure 9C shows that the *hlyA* single RNA was expressed under anaerobic conditions, but its level was reduced about threefold following oxygen exposure and treatment with $H_2O_2$. The low level of the polycistronic *hlyBA* transcript was also reduced under the same conditions. Taken together, these results suggest that an oxidative stress insult affects the control of *B. fragilis hlyBA* and *hlyA* transcription.

## DISCUSSION

In this study we describe the presence of hemolysins in *B. fragilis*, which is currently classified taxonomically as a nonhemolytic bacterium (18, 19, 53). The presence of at least 10 hemolysin orthologs in the *B. fragilis* genome suggests that they may play an important role in extraintestinal infections and possibly in the ecology and physiology of the intestinal tract. To the best of our knowledge, there is no previous report on the characterization of hemolysins in *B. fragilis* despite the fact that rare clinical strains have been reported to have a hemolytic phenotype in vitro (18, 19). Hemolysins/cytolysins are important toxins that lyse neutrophils and other host cells which can affect the immune response of the host and also provide a source of nutrients for the invading microorganism (13, 24, 38, 51). Hemolysins/cytolysins in many bacteria are not only considered potent virulence factors but are also cytolytic/bactericidal factors used to inhibit other prokaryotes and eukaryotes in highly competitive ecological systems (9). A classical example of the participation of cytolysin in both pathogenesis and ecological competitiveness is the two-peptide component cytolysin (bacteriocin) of the gastrointestinal tract inhabitant *Enterococcus faecalis*. Production of cytolysin by *E. faecalis* enhances lethality in human infections and animal models as well as providing a competitive advantage over other gram-positive bacteria in the intestinal tract (8, 9). In this regard, it is possible that the extensive number of hemolysins/cytolysins in *Bacteroides* species not only play a role in the infectious process but they also might contribute to their ability to dominate and take over the colonization of the human large intestinal tract.

The findings presented in this study indicate that hemolysins HlyA and HlyB are a two-component cytolysin. This is based

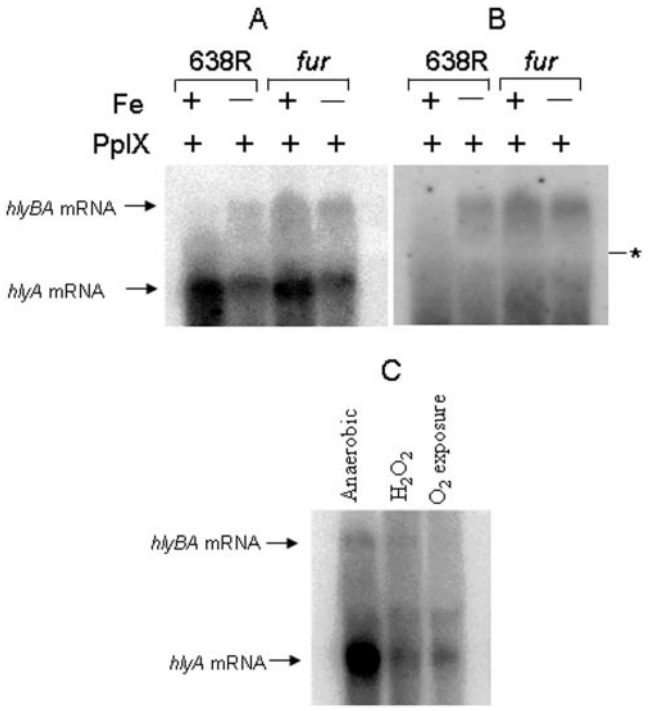


FIG. 9. Autoradiograph of Northern hybridization of total RNA. A and B) *B. fragilis* 638R and isogenic *fur* mutant were grown to mid-log phase in SDM containing 100 μM $FeSO_4$ for iron-replete conditions (+Fe) and SDM containing 50 μM 2,2′-bipyridyl and 100 μM desferrioxamine to impose iron-limiting conditions (−Fe). Protoporphyrin IX (PpIX) was added to the medium as a source of tetrapyrrole macrocycle. The probe was an internal fragment from *hlyA* (panel A) or *hlyB* (panel B). The positions of the *hlyA* and *hlyB* mRNA components are indicated. The asterisk depicts the 16S compression region on the filter membrane. (C) *B. fragilis* 638R was grown in SDM supplemented with 5 μg/ml hemin and exposed to different oxidative stress conditions. The probe was an internal fragment of *hlyA*.

on results showing that when both components are expressed together, there is a synergistic effect on hemolytic activity, and there is a protein-protein interaction. However, it is not known whether HlyA and HlyB protein complex formation occurs at the cell membrane or occurs prior to attachment on the eukaryotic cell surface. Though two-component hemolysins are produced by several bacteria (9, 25, 50, 52), *B. fragilis* HlyBA does not share homology with the classical *Serratia marcescens* family of two-component hemolysins (17) or bacterial pore-forming toxins of the RTX (repeat-in-toxin) exoprotein family (46). The mechanism of hemolytic activity of HlyBA is not known, but because HlyB has a conserved phospholipid/glycerol acyltransferase superfamily motif, we think that it might possess functional features within this group of enzymes involved in phospholipid biosynthesis (28, 48). Interestingly, the HlyB and HlyA peptides are homologous, respectively, to the acyltransferase and the hemolytic domains of hemolysins found in microorganisms such as *Vibrio anguillarum* and *W. succinogenes*. It is possible that a genetic mutation might have occurred in *Bacteroides* and in the closely related anaerobe *P. gingivalis* where HlyA and HlyB resulted from a split of a single peptide unit into two distinct peptides. The reverse phenomenon cannot be ruled out either. The reason for this is not known, but we presume that the two separate peptides may have other additional physiological properties directly interfering with bacterial physiology. In support of this is the fact that the *B. thetaiotaomicron hlyBA* genes are arranged in a transcriptional fashion similar to that in *B. fragilis* and the levels of *B. thetaiotaomicron hlyBA* mRNA are more abundant in early growth phase than the levels in later growth phase in vitro (44; NCBI/Gene Expression Omnibus (GEO) database repository/GEO database entry accession no. GSE2231).

Expression of HlyB and HlyA was shown to be differentially and coordinately regulated by oxygen and iron availability. The former represses *hlyBA* mRNA upon exposure to aerobic conditions, and our hypothesis is that HlyBA may be a potential virulence factor used by *B. fragilis* to injure and lyse host tissue cells in order to obtain essential nutrients in an extraintestinal anaerobic environment, such as an abscess. This is consistent with the fact that *B. fragilis* is able to proliferate after the establishment of anaerobic conditions at the site of infection (35, 36, 37, 39). In general, anaerobic conditions in the infected tissues are formed by the consumption of oxygen by facultative bacteria often found associated with anaerobes in polymicrobial infections (35, 36, 37, 39). The effects of oxygen limitation on hemolysin production and other virulence factors have been demonstrated in several pathogenic bacteria (10, 27, 47). For example, *E. faecalis* cytolysin/hemolysin $CylL_L$ and $CylL_S$ are up-regulated (8.6-fold) at the transcriptional level under anaerobic conditions compared to the levels in the presence of oxygen (10). The implications of anaerobic regulation of HlyBA in the pathogenicity of *B. fragilis* correlate nicely with the fact that the establishment of anaerobic infections in extraintestinal tissues follows the depletion of oxygen at the site of infection. Therefore, it is likely that HlyBA may contribute to *B. fragilis* pathogenicity in a low-oxygen and low-iron environment as discussed below.

Several studies have shown that *B. fragilis* is unable to synthesize protoporphyrin macrocycle and has an essential requirement for heme and nonheme iron (31, 45). This nutritional requirement correlates with the expression and role HlyBA might play to make possible bacterial access to iron and heme under the low-iron conditions encountered in host tissues. The production of hemolysins/cytolysins has been associated with the ability of pathogenic bacteria to obtain heme as a source of iron from lysed erythrocytes and other host cells (13). In agreement, iron-limiting conditions regulate the synthesis of hemolysins in many pathogenic bacteria (2, 3, 6, 23, 29). Thus, the iron-dependent regulation of *hlyBA* mRNA may help *B. fragilis* to overcome the iron-limiting conditions imposed by a host's iron-withholding mechanisms.

In conclusion, we showed in this study that *B. fragilis* encodes several functional hemolysins not previously characterized in this organism. Our studies on the synthesis and regulation of hemolysins may help us to understand *B. fragilis* pathogenicity and why this organism has a much greater potential to cause infections than any other anaerobic species that colonizes the human intestinal tract. In support of the hypothesis that hemolysins are important for *B. fragilis* virulence is a recent comparative genome analysis suggesting that the presence of hemolysin-like proteins and capsular polysaccharides may explain the difference in pathogenic

potentials between the two closely related species *B. fragilis* and *B. thetaiotaomicron* (22).


## ACKNOWLEDGMENTS

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